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**Abstract**

Mammalian color patterns are among the most recognizable characters found in nature and can have a profound impact on fitness. However, little is known about the mechanisms underlying their formation and subsequent evolution. Here we show that, in the African striped mouse (*Rhabdomys pumilio*), periodic dorsal stripes result from underlying differences in melanocyte maturation, which give rise to spatial variation in hair color, and we identify the transcription factor *Alx3* as a regulator of this process. In embryonic dorsal skin, patterned expression of *Alx3* foreshadows pigment stripes and acts to directly repress *Mitf*, a master regulator of melanocyte differentiation, giving rise to light-colored hair. Moreover, *Alx3* is also upregulated in the light stripes of chipmunks, which have independently evolved a similar dorsal pattern. Our results reveal a previously unappreciated mechanism for modulating spatial variation in hair color and provide new insight into the ways in which phenotypic novelty evolves.

A fundamental challenge in biology is to understand how repetitive morphologic structures develop and evolve. Periodic color patterns are a useful system with which to explore these questions because of their diversity, sophistication, and visual accessibility, and because the cellular, developmental, and molecular mechanisms that underlie spots and stripes in mammals are largely unknown. Traditional model organisms, such as laboratory mice (*Mus*), have been instrumental for identifying genes that regulate pigment cell production, melanin synthesis, and the pathways that alter the balance between two types of pigment: light pheomelanin and dark eumelanin[1-4]. However, it is unknown to what extent these pathways explain or even contribute to the remarkable array of pigment patterns seen in wild mammals. Here we take advantage of the naturally occurring coat pattern of the African striped mouse, *Rhabdomys pumilio* (Muridae), to gain insights into the processes underlying the formation and evolution of mammalian stripes, a striking and characteristic pattern that has evolved independently in many taxa, including ungulates, carnivores, rodents, marsupials, lagomorphs, and primates[3,5,6] and is thought to confer a fitness advantage in a range of vertebrates[7-10].

Striped mice are diurnal, social rodents distributed throughout southwest Africa[11] and whose dorsal coat is characterized by the presence of four dark and two light dorsal longitudinal stripes arranged in a dark-light-dark pattern (Fig. 1a). To understand how this stripe pattern is formed, we first characterized the distribution of hair and hair types across the body. In adult striped mice, hair can be classified into one of three phenotypic categories based on their individual pigment pattern – *light*: unpigmented shaft with a eumelanic base, *black*: completely eumelanic from tip to base, and *banded*: phaeomelanic (yellow) shaft and eumelanic (black) base (Fig. 1b and Extended Data Fig. 1a). We

47 quantified the proportion of each hair category in different regions along the dorsoventral
48 axis (middle stripe, dark stripe, light stripe, flank, and ventrum) and found that their
49 proportions differed: the middle stripe and flank had similar proportions of all three hair
50 types, but the light stripe contained mostly *light* hair, similar to what is seen in the
51 ventrum, and the dark stripe contained mostly *black* hair (Fig. 1b and Extended Data Fig.
52 1b). Thus, rather than by differences in pigment-type switching (pheomelanin vs.
53 eumelanin), variation between light and dark stripes is largely determined by changes in
54 the distribution of unpigmented (*light*) and eumelanic (*black*) hair.
55 To understand how such spatial differences arise, we examined skin development
56 of striped mice between embryonic and early postnatal stages, and observed stripe-like
57 changes in both hair length and skin color (Fig. 1c and Extended Data Fig. 2a-d). The
58 hair length changes first become apparent at embryonic day 19 (E19; Extended Data Fig.
59 2a, b), but do not persist in adult animals (Extended Data Fig. 2e). By contrast, the skin
60 color changes are first visible at E22, creating a stable arrangement and appearance that
61 persists as the animal grows (Fig. 1c). At birth, both phenomena are present and are
62 correlated with both morphology and gene expression (Extended Data Fig. 2c, d, f). Thus,
63 the patterning mechanisms that underlie adult stripes begin during embryogenesis and
64 can be visualized by changes in the organization of mesenchymal tissues.
65 By postnatal day 2 (P2), light and dark stripes exhibit similar levels of cell
66 proliferation and hair follicle densities (Extended Data Fig. 3). However, histological
67 sections of skin show that hair follicles from the light stripe display a striking reduction
68 in melanin deposition in the hair bulb relative to those from the dark stripe (Fig. 1d).
69 Although hair follicles from both light and dark stripes contain pigment cells, as revealed

by immunohistochemistry for the pigment cell marker KIT (Fig. 1e), those from light stripes exhibit less immunohistochemistry staining against MITF (Fig. 1f-h), a key transcription factor that promotes melanocyte differentiation and ultimately melanogenic gene expression[12]. The extent of KIT staining at the base of hair follicles was indistinguishable between dark and light stripes (Fig. 1i), despite the large differences in MITF staining and visible pigmentation. We used quantitative PCR (qPCR) to measure the level of gene expression for key melanogenic genes and observed that the light stripe had lower expression of both *Tyrosinase* (*Tyr*) and *Tyrosinase-related protein1* (*Tyrp1*) (Fig. 1j). Taken together, these results suggest that the differences in the proportions of *light* and *black* hair in light and dark stripes, respectively, are explained largely by differences in levels of *Mitf*, extent of melanocyte differentiation, and amount of melanogenic gene expression.

**Spatial differences in gene expression**

We used RNA-Seq to carry out an unbiased survey of transcriptome differences that correlate with stripe identity. Three different skin regions (light stripe, dark stripe, and flank) from each of three individuals were examined at four stages (E19, E22, P0, P2; $n$ = 12; 36 libraries). (For E19, we used hair length as a proxy to mark and isolate incipient pigmentation stripes; Extended Data Fig. 2). Our initial analyses were carried out using, as a reference, either the *Mus* genome or a striped mouse transcriptome that we assembled and annotated in-house. The results from the two approaches exhibited considerable overlap but alignment to the *de novo* transcriptome reference captured a richer and more comprehensive differential signature (Extended Data Fig. 4a-c), and is

93 described in what follows. Of more than 17000 genes that we annotated in the striped
94 mouse transcriptome, 1062 exhibited significant differential expression between two
95 regions (false discovery ratio [FDR] < 0.1, using the negative binomial generalized linear
96 model implemented in DESeq2, for which both stage and region are considered as
97 factors). The largest number of differentially expressed genes between regions was
98 observed for the flank vs. the dark or the light stripe (Extended Data Fig. 4d-f), which
99 likely reflects a difference in body region or tissue composition rather than a color
100 pattern-specific difference. Among 36 genes significantly upregulated in the dark stripe
101 vs. the light stripe, there is an obvious signature of melanocyte pigment production (*Tyr,*
102 *Tyrp1, Mc1r, Oca2, Gpr143, Trpm1*; Fig. 2a and Supplementary Table 1a). Several of
103 these genes are direct targets of *Mitf*, but we did not observe a difference in *Mitf* mRNA
104 levels between dark and light stripe skin, probably because *Mitf* is also expressed in non-
105 pigmentary skin cells outside the hair follicle.

106 Among 28 genes significantly upregulated in the light vs. the dark stripe, a clear
107 functional signature did not emerge; however, our attention was drawn to *Alx3*, which
108 encodes a paired-class *aristaless*-like homeoprotein that has previously been implicated
109 in fate specification of mesenchymal tissues[13-16]. As described below, the temporal and
110 spatial expression of *Alx3* make it a strong candidate for regulating color differences
111 among stripes. Furthermore, *Alx3* showed the highest fold differences in transcript
112 abundance relative to dark stripe and the flank (6.73 and 4.32-fold, FDR $1.16*10^{-17}$ and
113 $4.09*10^{-11}$ respectively; Fig. 2a, Extended Data Fig. 4f, and Supplementary Table 1a, b).

114 To further investigate a potential role of *Alx3* in stripe patterning, we examined its
115 spatial and temporal expression during skin development and stripe formation.

116 Quantitative RT-PCR showed that *Alx3* mRNA is elevated in the incipient light vs. dark
117 stripes at E19, the difference increases at E22, and then remains but is reduced at P0 (Fig.
118 2b). This pattern contrasts with that observed for other pigmentation-related genes
119 including *Asip*, *Edn3*, *Tyr*, and *Tyrp1* (Extended Data Fig. 5) for which differential
120 expression is not apparent until E22 or later.
121
122 **_Alx3_ expression during development**
123 Using *in situ* hybridization, we examined the expression of *Alx3* during the early
124 stages of skin development. Previous work on *Alx3* during embryogenesis (E10.5-E12.5)
125 in *Mus* showed expression in neural crest-derived mesenchyme and in lateral plate
126 mesoderm[13]. In sections from E13.5 and E15.5 *Mus* embryos, we confirmed this pattern
127 of expression, observing *Alx3* mRNA in lateral mesenchyme at E13.5, extending to and
128 predominating in ventral mesenchyme by E15.5 (Fig. 2c). A similar pattern is present in
129 striped mice (Fig. 2d); however, striped mouse embryos show an additional domain of
130 *Alx3* expression in the developing dorsal skin (red arrowheads in Fig. 2d), which
131 corresponds anatomically with the future position of the stripe domain. At these early
132 (E15) stages, ALX3 is primarily expressed in epithelial cells, as indicated by combined
133 immunohistochemistry with E-cadherin, an epidermal compartment marker (Fig. 2e). At
134 P0, when *Alx3* mRNA remains elevated in light vs. dark stripe skin, immunostaining for
135 ALX3 reveals cells in developing dorsal hair bulbs (Fig. 2f). Combined immunostaining
136 with antisera for S100 (Fig. 2g), a marker for neural crest-derived cells[17], indicates that
137 hair bulb expression of ALX3 includes melanocytes as well as keratinocytes (Fig. 2h).
138 Taken together, these results demonstrate that establishment of a cellular compartment

corresponding to the stripe domain occurs early during skin development, implicate *Alx3* as a participating and/or responding factor in pattern establishment, and suggest multiple pathways—a direct effect on pigment cells and/or an indirect effect on hair bulb keratinocytes—through which alterations of melanocyte *Mitf* expression and pattern implementation may take place.

**_Alx3_ has a melanocyte autonomous effect**

To further investigate the potential relationship between *Alx3*, *Mitf*, and pigment production, we carried out both gain- and loss-of-function perturbation experiments in cultured cells. First, a lentiviral construct in which *Alx3* and a *GFP* reporter are driven by a PGK promoter (LV-*Alx3*:GFP; Extended Data Fig. 6a) was transduced into B16-F1 cells, a *Mus* melanocyte cell line that expresses *Alx3* (Extended Data Fig. 6b); control cells were transduced with the same construct but lacking *Alx3* (LV-GFP)[18] (Extended Data Fig. 6a). We found that cells carrying the full length *Alx3* construct (LV-*Alx3*:GFP) exhibited a marked decrease in mRNA levels of *Mitf* and *Silver,* a key melanogenesis gene and *Mitf* target (Extended Data Fig. 6c). Second, for loss-of-function experiments, we used short hairpin RNAs (shRNAs) against *Alx3.* Three of the four shRNA lentiviral constructs tested (shRNA1, 2, and 3) caused *Alx3* mRNA levels to decrease relative to cells containing a scrambled shRNA control (Extended Data Fig. 6d). The same three constructs caused *Mitf* and *Silver* mRNA levels to increase. Thus, these experiments together show that *Alx3* can negatively regulate *Mitf* in a cell autonomous fashion.

To investigate a potential non-cell autonomous effect of *Alx3* on *Mitf* expression, we transduced primary *Mus* keratinocytes with LV-*Alx3*:GFP or LV-GFP and co-cultured them with wildtype B16-F1 melanocytes using a cell culture insert system (Extended

Data Fig. 7a). Melanocytes exposed to keratinocytes stably expressing LV-*Alx3*:GFP did not differ in their *Mitf*, *Tyr*, or *Silver* mRNA levels, relative to those co-cultured with LV-GFP expressing keratinocytes (Extended Data Fig. 7b). In addition, we obtained a similar response when wildtype melanocytes were co-cultured with LV-*Alx3*:GFP transduced melanocytes (Extended Data Fig. 7c, d). Taken together, these results reveal a reciprocal relationship between *Alx3* and *Mitf*, in which expression of *Mitf* mRNA and its melanogenic gene targets can either be up- or down-regulated in response to inhibition or overexpression, respectively, of *Alx3* mRNA. Our results further indicate that regulation of *Mitf* expression and melanogenic gene expression by *Alx3* occurs via a melanocyte-autonomous process.

**_Alx3_ decreases melanogenesis _in vivo_**

We next examined the effect of *Alx3 in vivo* using the same lentiviral constructs described above and ultrasound-assisted *in utero* injections into E8.5 mouse embryos. At this stage, prior to neural tube closure, injected lentivirus infects skin epidermis and cells originating from the neural crest, including melanocyte precursors (Fig. 3a, b). We observed GFP expression in cells in the center of the hair follicle as well as the upper periphery; co-staining with KIT confirmed that many/most of the GFP-positive cells in the center of the follicle were melanocytes (Fig. 3b).

Hair follicles from mice sampled at P4, a stage in which melanin synthesis is active[2], that were transduced with LV-*Alx3*:GFP had a marked reduction in the number of $MITF^{+}GFP^{+}$ cells compared to the control, as revealed by immunohistochemistry (Fig 3c-i). In addition, LV-*Alx3*:GFP transduced melanocytes, isolated by FACS, showed a

185 decrease in mRNA levels of melanin synthesis markers (*Tyrp1*, *Tyr*, and *Silver*) relative
186 to the control (Fig. 3j). To determine whether the effect of *Alx3* over-expression reduced
187 the number of melanocytes or just their expression of *Mitf*, we performed
188 immunohistochemistry against SOX10, a melanocyte marker upstream of *Mitf*[12,19-21], and
189 found that the number of $SOX10^{+}GFP^{+}$ cells in LV-*Alx3*:GFP and control samples did
190 not differ (Extended Data Fig. 8a-g). Furthermore, we did not detect a difference in the
191 number of keratinocytes, as determined by counts of $K14^{+}GFP^{+}$ cells inside hair follicles,
192 or in hair follicle density (Extended Data Fig. 8h-o). The ability of elevated levels of *Alx3*
193 to suppress expression of *Mitf* and melanocyte differentiation in experiments with *Mus*
194 recapitulates the differences seen between the light and dark stripes of striped mice.
195
196 ***Alx3* directly represses *Mitf***
197 The histological and genomic data point to several pathways that contribute to
198 stripe differences, in which an *Alx3*-*Mitf* connection may play an early and predominant
199 role. We next used *in vivo* and *in vitro* protein-DNA interaction experiments to gain
200 additional insight into how *Alx3* downregulates *Mitf*. *Alx3* can selectively bind to a DNA
201 consensus sequence containing a TAAT motif[22]. The *Mitf* gene contains nine distinct
202 promoters, of which one, 1M, is selective to melanocytes and prominently active in these
203 cells[12]. In a ~1.5kb region upstream of the *Mus Mitf* M transcriptional initiation site,
204 including regions known to be relevant for transcription factor binding[23], we identified
205 ten candidate TAAT binding sites conserved between striped mice and *Mus*, four of
206 which are also conserved across mammals (labeled 1-10; Fig. 4a and Extended Data Fig.
207 9). Electrophoretic mobility shift assays (EMSA) with nuclear extracts of B16-F1 cells

208 revealed that binding sites 3, 5, and 10 generated sequence-specific DNA-protein
209 complexes (Extended Data Fig. 10a,b). The addition of ALX3 antiserum[24] disrupted the
210 formation of these complexes, demonstrating that ALX3 binds to the oligonucleotide
211 probes for these three sites (Extended Data Fig. 10a).

212 We then carried out chromatin immunoprecipitation (ChIP) assays on B16-F1
213 cells and found that *Mitf* promoter sequences that contain candidate binding sites 3, 5,
214 and 10 were selectively amplified by qPCR from chromatin immunoprecipitated with the
215 anti-ALX3 antiserum, but not with control non-immune serum (Fig. 4b). Promoter
216 sequences from the *Tyr* gene, used as a control, were not amplified from the anti-ALX3
217 immunoprecipitated chromatin (Fig. 4b). Thus, ALX3 binds to evolutionarily conserved
218 sequences immediately upstream of the melanocyte *Mitf* promoter both *in vitro* and *in*
219 *vivo*.

220 We also tested the effect of *Alx3* overexpression on *Mitf* promoter activity in B16-
221 F1 cells. Using a luciferase reporter cloned downstream of *Mitf* promoter sequences,
222 overexpression of *Alx3* caused a decrease in luciferase expression relative to GFP
223 overexpression (Extended Data Fig. 10c). Furthermore, when we mutated the TAAT
224 motifs relevant for ALX3 interactions as identified by our EMSA experiments (i.e., sites
225 3, 5 and 10), luciferase activity between GFP and *Alx3* expressing cells did not differ,
226 indicating that *Alx3* could no longer suppress expression (Extended Data Fig. 10c). As a
227 control, we mutated candidate binding site 1, which did not show binding in our EMSA
228 experiments, and found that *Alx3* transfected cells had a decrease in luciferase activity
229 relative to GFP transfected cells, similar to what was seen with the wild-type *Mitf*
230 promoter (Extended Data Fig. 10c). Together these results provide compelling evidence

231 that that *Alx3* represses *Mitf* in melanocytic cells by binding directly to specific sequences
232 in its promoter.
233
234 **Convergent evolution of striped rodents**
235 Color patterns in striped mice are very similar to, but independently evolved from,
236 those seen in Eastern chipmunks (*Tamias striatus*), a sciurid rodent which shares a last
237 common ancestor with murids about 70mya[25-27] (Fig. 5a). In skin biopsies from adult
238 Eastern chipmunks, we measured expression of *Alx3* using qPCR and found that mRNA
239 levels are higher in the light stripe relative to the dark stripe and flank (Fig. 5b). We also
240 measured expression of *Asip* and *Edn3*, which encode paracrine regulators of pigment-
241 type switching, and compared the results in striped mice (Extended Data Fig. 6a, b) to
242 those in chipmunks (Fig. 5b). Overall, *Asip* mRNA levels are elevated in light areas (the
243 light stripe, flank, and ventrum in striped mice and the flank and the ventrum in
244 chipmunks), whereas *Edn3* mRNA levels are elevated in dark areas (the dark stripes in
245 both species). Thus, *Alx3*-mediated repression of *Mitf* in melanocytes and paracrine
246 regulation of pigment-type switching are common developmental mechanisms used
247 repeatedly during mammalian evolution to bring about stripe patterns.
248
249 **Discussion**
250 In non-mammalian vertebrates, mechanisms that underlie color patterns depend
251 on specialized types of pigment cells that become organized into specific arrangements
252 during development[3,28-30]. But in mammals, there exists only a single type of pigment
253 cell, the melanocyte, and, in general, localized differences in color are brought about by

heterogeneity of gene expression rather than heterogeneity of cellular distribution[1,3,31]. For stripes and spots in felids, pigment-type switching controlled by *Asip* and *Edn3* is the major determinant of color pattern[32], but for dorsal stripes in striped mice, suppression of melanocyte development is a main driver of color pattern. Indeed, *Alx3*-induced inhibition of *Mitf* expression without loss of melanocytes represents a previously unrecognized evolutionary tool for the local modulation of color pattern.

In striped mice and chipmunks, both *Alx3*-mediated repression of *Mitf* and pigment-type switching modulate hair color. For example, increased expression of both *Asip* and *Alx3* distinguish the ventrum from the flank and increased expression of *Edn3* distinguishes the dark stripes from surrounding regions. This interplay between melanocyte-autonomous (*Alx3*-dependent) and non-autonomous (paracrine regulation of pigment-type switching) pathways may help explain patterns composed of multiple intensities of pigment (e.g., pattern elements that are almost devoid of pigment [*Alx3*-mediated repression of *Mitf*] mixed with elements that are yellow or orange [pigment-type switching]) (Fig. 5c). Future efforts, directed towards developing gene-editing approaches in striped mice and chipmunks, will aim to dissect the relative contribution of each pathway to the resulting phenotype.

From an evolutionary perspective, we note that laboratory mice, striped mice, and chipmunks all express *Alx3* in ventral skin, but only the latter two species upregulate *Alx3* in their dorsum. While *Alx3* and *Asip*[33,34] contribute to pale-colored ventrums, we hypothesize that *Alx3* was subsequently co-opted and expressed in an additional dorsal domain, ultimately giving rise to light-colored stripes. It is not yet clear if the developmental cues that direct *Alx3* expression and stripe formation in striped mice and

chipmunks originate from an organizing center, such as the neuroectoderm, or arise spontaneously (e.g., through a reaction-diffusion mechanism), but it is possible that the same principles operate broadly across mammalian taxa. Finally, although striped mice and chipmunks share a similar dark-light-dark stripe pattern, the location of the stripes along the dorso-ventral axis differs considerably, with stripes in chipmunks situated more laterally than in striped mice. In addition, stripe number varies widely among rodents, from the single-striped grass mouse (*Lemniscomys rosalia*) to the thirteen-lined ground squirrel (*Ictidomys tridecemilineatus*). From this perspective, a detailed understanding of the spatial regulation of *Alx3* in rodents, and other striped mammals, will lead to exciting insights into the evolution of gene regulation and the developmental basis of evolutionary novelty.

**References**


1. Barsh, G. S. The genetics of pigmentation: from fancy genes to complex traits. *Trends Genet.* **12,** 299–305 (1996).
2. Jackson, I. Molecular and developmental genetics of mouse coat color. *Annu. Rev. Genet.* **28,** 189–217 (1994).
3. Mills, M. G. & Patterson, L. B. Not just black and white: Pigment pattern development and evolution in vertebrates. *Semin. Cell Dev. Biol.* **20,** 72–81 (2009).
4. Candille, S. I. *et al.* Dorsoventral patterning of the mouse coat by *Tbx15*. *Plos Biol.* **2,** e3 (2004).
5. Morris, D. *Animal Watching. A Field Guide to Animal Behavior*. (1990).

6. Cloudsley-Thompson, J. L. Multiple factors in the evolution of animal coloration. *Naturwissenschaften* **86,** 123–132 (1999).

7. Caro, T., Izzo, A., Reiner, R. C., Walker, H. & Stankowich, T. The function of zebra stripes. *Nature Comms.* **5,** 1–10 (2014).

8. Brodie, E. D., III. Correlational selection for color pattern and antipredator behavior in the garter snake *Thamnophis ordinoides*. *Evolution* **46,** 1284–1298 (2005).

9. King, R. B. Color pattern polymorphism in the Lake Erie water snake, *Nerodia sipedon insularum*. *Evolution* **41,** 241–255 (1987).

10. Caro, T. The adaptive significance of coloration in mammals. *BioScience* **55,** 125–136 (2005).

11. Schradin, C. *et al.* Social flexibility and social evolution in mammals: a case study of the African striped mouse (*Rhabdomys pumilio*). *Mol. Ecol.* **21,** 541–553 (2012).

12. Steingrimsson, E., Copeland, N. G. & Jenkins, N. A. Melanocytes and the Microphthalmia transcription factor network. *Annu. Rev. Genet.* **38,** 365–411 (2004).

13. ten Berge, D. *et al.* Mouse *Alx3*: An aristaless-like homeobox gene expressed during embryogenesis in ectomesenchyme and lateral plate mesoderm. *Dev. Biol.* **199,** 11–25 (1998).

14. Beverdam, A., Brouwer, A., Reijnen, M., Korving, J. & Meijlink, F. Severe nasal clefting and abnormal embryonic apoptosis in *Alx3*/*Alx4* double mutant mice. *Development* **128,** 3975–3986 (2001).

15. Twigg, S. R. F. *et al.* Frontorhiny, a distinctive presentation of frontonasal dysplasia caused by recessive mutations in the *Alx3* homeobox gene. *Am. J. Hum. Genet.* **84,** 698–705 (2009).

16. Lakhwani, S., García-Sanz, P. & Vallejo, M. *Alx3*-deficient mice exhibit folic acid-resistant craniofacial midline and neural tube closure defects. *Dev. Biol.* **344,** 869–880 (2010).

17. Peters, E. M. J., Tobin, D. J., Botchkareva, N., Maurer, M. & Paus, R. Migration of melanoblasts into the developing murine hair follicle is accompanied by transient c-Kit expression. *J. Histochem. Cytochem.* **50,** 751–766 (2002).

18. Beronja, S., Livshits, G., Williams, S. & Fuchs, E. Rapid functional dissection of genetic networks via tissue-specific transduction and RNAi in mouse embryos. *Nat. Med.* **16,** 821–827 (2010).

19. Lee, M., Goodall, J., Verastegui, C., Ballotti, R. & Goding, C. R. Direct regulation of the Microphthalmia promoter by *Sox10* links Waardenburg-Shah syndrome (WS4)-associated hypopigmentation and deafness to WS2. *J. Biol. Chem.* **275,** 37978–37983 (2000).

20. Potterf, S. B., Furumura, M., Dunn, K. J., Arnheiter, H. & Pavan, W. J. Transcription factor hierarchy in Waardenburg syndrome: regulation of MITF expression by SOX10 and PAX3. *Hum. Genet.* **107,** 1–6 (2000).

21. Elworthy, S., Lister, J. A., Carney, T. J., Raible, D. W. & Kelsh, R. N. Transcriptional regulation of *Mitf* accounts for the *Sox10* requirement in zebrafish melanophore development. *Development* **130,** 2809–2818 (2003).

22. Perez-Villamil, B., Mirasierra, M. & Vallejo, M. The homeoprotein *Alx3* contains

discrete functional domains and exhibits cell-specific and selective monomeric binding and transactivation. *J. Biol. Chem.* **279,** 38062–38071 (2004).

23. Levy, C., Khaled, M. & Fisher, D. E. MITF: master regulator of melanocyte development and melanoma oncogene. *Trends. Mol. Med.* **12,** 406–414 (2006).

24. García-Sanz, P., Fernández-Pérez, A. & Vallejo, M. Differential configurations involving binding of USF transcription factors and *Twist1* regulate *Alx3* promoter activity in mesenchymal and pancreatic cells. *Biochem. J.* **450,** 199–208 (2013).

25. Meredith, R. W. *et al.* Impacts of the Cretaceous terrestrial revolution and KPg extinction on mammal diversification. *Science* **334,** 521–524 (2011).

26. Reis, dos, M. *et al.* Phylogenomic datasets provide both precision and accuracy in estimating the timescale of placental mammal phylogeny. *Proc. Roy. Soc. Lond. B. Bio.* **279,** 3491–3500 (2012).

27. Huchon, D. *et al.* Multiple molecular evidences for a living mammalian fossil. *Proc. Natl. Acad. Sci. USA.* **104,** 7495–7499 (2007).

28. Rawls, J. F., Mellgren, E. M. & Johnson, S. L. How the zebrafish gets its stripes. *Dev. Biol.* **240,** 301–314 (2001).

29. Parichy, D. M. Pigment patterns: fish in stripes and spots. *Curr. Biol.* **13,** R947–950 (2003).

30. Singh, A. P. & Nüsslein-Volhard, C. Zebrafish stripes as a model for vertebrate colour pattern formation. *Curr. Biol.* **25,** R81–92 (2015).

31. Jackson, I. *et al.* Genetics and molecular biology of mouse pigmentation. *Pig. Cell Res.* **7,** 73–80 (1994).

32. Kaelin, C. B. *et al.* Specifying and sustaining pigmentation patterns in domestic

and wild cats. *Science* **337,** 1536–1541 (2012).

33. Vrieling, H., Duhl, D. M., Millar, S. E., Miller, K. A. & Barsh, G. S. Differences in dorsal and ventral pigmentation result from regional expression of the mouse agouti gene. *Proc. Natl. Acad. Sci. USA*. **91,** 5667–5671 (1994).

34. Manceau, M., Domingues, V. S., Mallarino, R. & Hoekstra, H. E. The developmental role of *Agouti* in color pattern evolution. *Science* **331,** 1062–1065 (2011).

35. Schneider, C. A., Rasband, W. S. & Eliceiri, K. W. NIH Image to ImageJ: 25 years of image analysis. *Nat. Methods* **9,** 671–675 (2012).

36. McCloy, R. A. *et al.* Partial inhibition of *Cdk1* in G 2phase overrides the SAC and decouples mitotic events. *Cell Cycle* **13,** 1400–1412 (2014).

37. Spandidos, A., Wang, X., Wang, H. & Seed, B. PrimerBank: a resource of human and mouse PCR primer pairs for gene expression detection and quantification. *Nucleic Acids Res.* **38,** D792–D799 (2009).

38. Livak, K. J. & Schmittgen, T. D. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* **25,** 402–408 (2001).

39. Henrique, D. *et al.* Expression of a Delta homologue in prospective neurons in the chick. *Nature* **375,** 787–790 (1995).

40. Mallarino, R. *et al.* Two developmental modules establish 3D beak-shape variation in Darwin's finches. *Proc. Natl. Acad. Sci. USA* **108,** 4057–4062 (2011).

41. Moffat, J. *et al.* A lentiviral RNAi library for human and mouse genes applied to an arrayed viral high-content screen. *Cell* **124,** 1283–1298 (2006).

392 42. Kent, W. J. *et al.* The human genome browser at UCSC. *Genome Res.* **12,** 996–
393 1006 (2002).
394 43. Schwartz, S. *et al.* PipMaker---A web server for aligning two genomic DNA
395 sequences. *Genome Res.* **10,** 577–586 (2000).
396 44. Brudno, M. *et al.* LAGAN and Multi-LAGAN: efficient tools for large-scale
397 multiple alignment of genomic DNA. *Genome Res.* **13,** 721–731 (2003).
398 45. Schreiber, E., Matthias, P. & Müller, M. M. Rapid detection of octamer binding
399 proteins with 'mini extracts', prepared from a small number of cells. *Nucleic Acids*
400 *Res.* **17,** 6419 (1989).
401 46. Mirasierra, M. & Vallejo, M. Glucose-dependent downregulation of glucagon gene
402 expression mediated by selective interactions between ALX3 and PAX6 in mouse
403 alpha cells. *Diabetologia* **1,** 1–10 (2016).
404 47. de la Serna, I. L. *et al.* The microphthalmia-associated transcription factor requires
405 SWI/SNF enzymes to activate melanocyte-specific genes. *J. Biol. Chem.* **281,**
406 20233–20241 (2006).
407

408 **Figure legends**

409 **Figure 1. Phenotypic characterization. a,** Coat pattern of African striped mice. **b,**
410 Proportion of each hair type in adults ($n$ = 5; SEM). **c,** Dorsal pattern at E22, P0, and P2.
411 **d-f,** Skin sections from P2 dark and light stripes showing hematoxilin and eosin stain (**d**),
412 and immunohistochemistry for KIT (**e**) and MITF (**f**). Brightfield images (**e, f**) depict
413 pigment. **g-h,** Number of detectable $MITF^+$ cells (**g**; dark vs. light, two-tailed $t$ test; $n$ =
414 60) and their fluorescence intensity (**h**; dark vs. light, two-tailed $t$ test; $n$ = 34). **i,** Extent

of KIT stain (dark vs. light, two-tailed *t* test; $n = 20$, $P = 0.5429$). **j,** Quantitative PCR of melanogenic genes at P2 (ANOVA followed by a Tukey-Kramer test; $n = 4$); Scale bars (**d, k**) 100 μm; (**e, f**) 50 μm. *$P < 0.05$, **$P < 0.01$; ***$P < 0.001$.

**Figure 2. *Alx3* is a candidate for regulating spatial differences in hair color. a,** Differential expression (DE) of transcripts in light vs. dark stripe ($n = 145$; FDR < 0.1); higher expression in light (yellow) or dark stripe (blue). Known pigmentation genes are labeled, those with DE ($n = 9$; dark yellow, dark blue) and without DE ($n = 8$; pink). **b,** Quantitative PCR of *Alx3* at three stages (ANOVA followed by a Tukey-Kramer test; $n = 4$; statistically significant differences ($P < 0.05$) indicated by different letters. Red bars show the mean. **c, d,** *In situ* hybridization shows *Alx3* lateral and ventral mesenchyme expression in *Mus* (**c**) and striped mouse (**d**) and dorsal expression unique to striped mice (red arrows). **e-h,** Immunohistochemistry for ALX3 (arrowheads) and E-cadherin at E15 (**e**) and ALX3 (**f**) and S100 (**g**) in P0 dorsal hair follicles. Arrowheads in (**h**) show colocalization; nt: neural tube, end: endoderm. Scale bars in (**c, d**) 200 μm, (**e-h**) 50 μm.

**Figure 3. *Alx3* decreases melanin synthesis *in vivo*. a, b,** Injections at E8.5 allow stable transduction of several cell types, including melanocytes (arrowheads). **c-h,** Hair follicles from samples injected with LV-GFP control (**c-e**) and LV-*Alx3*:GFP (**f-h**) depicting immunohistochemistry for MITF (**c, f**), virus transduced cells (**d, g**), and merged images showing $MITF^+GFP^+$ cells (**e, h**) (arrowheads). **i,** Number of detectable $MITF^+$ cells (LV-*Alx3*:GFP vs. LV-GFP, two-tailed *t* test; $n = 60$, ***$P < 0.001$). **j,** Quantitative PCR

438 shows mRNA levels of melanogenic genes (LV-GFP vs. LV-*Alx3*:GFP, two-tailed *t* test;
439 $n = 3$, ***$P < 0.001$).
440
441 **Figure 4. *Alx3* binds to the *Mitf* promoter directly. a,** Approximate location of putative
442 *Alx3* binding sites (labeled 1-10), conserved in *Mus* and striped mouse (red circles) and
443 across mammals (orange circles), along the 1.5kb region of the *Mitf* M promoter. **b,**
444 ChIP-qPCR assays showing amplification of *Mitf* chromatin corresponding to different
445 regions of the promoter immunoprecipitated with an anti-ALX3 antibody or with control
446 NRS or IgG (Anti-ALX3 vs. NRS or IgG, two-tailed *t* test; $n = 4$, *$P < 0.05$).
447
448 **Figure 5. Hair color patterning mechanisms in rodents. a,** Chipmunks independently
449 evolved a dorsal pattern that resembles striped mice. Photo: Joel Sartore/National
450 Geographic Photo Ark. **b,** Quantitative PCR shows *Alx3*, *Asip*, and *Edn3* mRNA levels
451 along the dorsoventral axis. Differences evaluated by ANOVA followed by a Tukey-
452 Kramer test; $n = 4$; statistically significant differences ($P < 0.05$) are indicated by
453 different letters. Red bars show the mean. **c,** Combination of a melanocyte autonomous
454 pathway, mediated by *Alx3*, and a non-autonomous pathway, mediated by paracrine
455 factors (*Edn3* and *Asip*), may explain variation in rodent pigmentation patterns,
456 specifically, and mammals, generally.
457
458 **Methods**
459 **Striped mouse breeding colony**

460 F10 descendants of wild-derived striped mice (*Rhabdomys pumilio*, originating
461 from Goegap Nature Reserve, South Africa S29 41.56, E 18 1.60) were obtained from a
462 captive colony at the University of Zurich (Switzerland) and now maintained at Harvard
463 University. They are kept at a 16:8 light dark cycle and given food *ad libitum*.
464 Developmental stages were inferred from morphological similarities with *Mus* embryos.
465 Harvard University's IACUC committee approved all experiments.
466
467 **Phenotypic characterization**
468 *Adults:* We identified three main hair types based on their individual pigment
469 pattern: *black*, *banded*, and *light*. To characterize pigment pattern along the dorsoventral
470 axis, we quantified the proportion of each of these hair types in 1mm hair plugs taken
471 from each dorsal stripe, the flank and the ventrum of five adult mice. In addition, we
472 scored the number of guard, awl, and zigzag hair found in each region. To determine hair
473 length, we placed hairs from the hair plugs on microscope slides, mounted them with
474 glycerol, and measured their length using Axiovision Microscopy Software (Zeiss).
475 *Embryos and Pups:* We fixed embryos, dissected the skin, and mounted it on
476 glass slides (dermal side up). For estimating hair follicle density in pups, we detached a
477 portion of each stripe from the muscle, while leaving the ends attached, embedded
478 samples in OCT (Fisher Scientific), cryosectioned them coronally, and stained them with
479 Hematoxilin and Eosin. This technique allowed us to count individual hair follicles and
480 assign them to the specific region to which they belonged (light stripes, dark stripes, or
481 flank). We counted the number of hair follicles and estimated surface area of the tissue
482 using ImageJ[35]. Since our phenotypic characterization (Fig. 1b and Extended Data Figs.

483 1b and 3b) and gene expression patterns determined by qPCR (Figs. 1j and 2b) showed
484 no differences between the two dark stripes, we carried out most analyses with dark stripe
485 1 (closest to the midline). We manually quantified the number of $MITF^{+}$ cells per hair
486 follicle, as detected with the antibody. For quantification of MITF fluorescence, we
487 obtained images from hair follicles in the light and dark stripe, outlined stained cells, and
488 measured the integrated density using ImageJ[35]. To obtain the corrected total cell
489 fluorescence (CTCF), we multiplied the area of each selected cell by the mean
490 fluorescence of the background readings and subtracted that value from the integrated
491 density of stained cells[36]. To quantify the extent of KIT staining, we obtained images
492 from hair follicles in the light and dark stripe, delineated the hair bulb area, and measured
493 the proportion of the area that was stained with $KIT^{+}$ using ImageJ. Data were obtained
494 from three pups and three embryos. All counts were done blind. Statistical differences
495 were determined using two-tailed *t* tests or ANOVA (sample sizes and statistical tests
496 used are indicated in figure legends).
497
498 **Cell proliferation**
499 We injected the peritoneum of striped mouse pregnant females with 10 µg/g of
500 EdU (5-ethynyl-2'-deoxyuridine) two days prior to birth and collected pups at P2. To
501 reveal proliferating cells, we microdissected dark and light stripes, embedded them in
502 OCT for posterior cryosectioning, and used the Click-iT EdU Imaging kit (ThermoFisher
503 Scientific), following the manufacturers protocol. We counted proliferating cells in the
504 epidermis and hair follicles from pictures of light and dark stripes. Data were obtained

505 from three individuals. All counts were done blind. Statistical differences were
506 established using two-tailed *t* tests (sample sizes are provided in the figure legend).
507
508 **Immunohistochemistry**
509 Striped mouse embryos were fixed in 4% PFA, embedded in OCT/sucrose, and
510 sectioned using a cryostat (CM 3050S, Leica). We performed immunohistochemistry
511 using anti-MITF (Abcam 80651; 1:100), anti-ALX3 (Abcam 64985; 1:500), anti-KIT
512 (DAKO A4502; 1:1000), anti-E-cadherin (Millipore ECCD-2; 1:200), anti-S100 (Abcam
513 4066; 1:200), and anti-SOX10 (Abcam 27655; 1:100). We visualized reactions with
514 Alexa dye conjugated secondary antibodies (Molecular Probes) at 1:500 dilution in 3%
515 BSA/PBS/Tween or with Biotinylated Goat Anti Rabbit (Jackson Labs) and TSA (Perkin
516 Elmer). For controls, we incubated sections with PBS instead of primary antibodies, but
517 no specific cellular staining was observed. Cell nuclei were stained with DAPI (Southern
518 Biotech). We examined sections using a LSM 700 confocal microscope and an A1
519 Imager (Zeiss). All pictures are representative of at least three individuals.
520
521 **Quantitative PCR (qPCR)**
522 We separated the skin from the muscle and microdissected skin tissue
523 corresponding to different regions (i.e., dark stripe 1, light stripe, dark stripe 2, flank, and
524 ventrum) at the different time points indicated throughout the text. We then extracted
525 total RNA using the Fibrous Tissue RNeasy kit (Qiagen), which included a DNAse on
526 column treatment. Using qScript cDNA SuperMix (Quanta BioSciences), we generated
527 complementary DNA (cDNA) and then performed qPCR using PerfeCTa SYBR Green

FastMix (Quanta BioSciences). We used 40 cycles of amplification on an Eppendorf Mastercycler. For analysis of striped mice, we designed primers along sites that were conserved across mice and rats. For chipmunk samples, we designed primers along sites that were conserved across mice and thirteen-lined ground squirrels (all primers sequences are listed in Supplementary Table 2). For measurements of *Mitf* expression in *Mus* samples, we used validated qPCR primers from the PrimerBank database[37]. We assayed gene expression in triplicate for each sample and normalized the data using the housekeeping gene *β-actin*. Samples used for qPCR correspond to different individuals than those used for RNA-seq analysis. We analyzed data from all qPCR experiments using the comparative CT method[38], and established statistical significance of expression differences using either ANOVA followed by a Tukey-Kramer test or two-tailed *t* tests (sample sizes and specific statistical tests used are given in each figure legend).

**RNA sequencing**

For each of the time points described in the text, we dissected skin tissue (dark stripe 1, light stripe, flank) and extracted RNA as indicated for qPCR. We used RNA from three different regions (light stripe, dark stripe, and flank) from each of three individuals for four different stages (E19, E22, P0, P2; $n = 12$; 36 libraries in total). We prepared cDNA libraries for each sample using Illumina's TruSeq RNA Library Preparation Kit v2. We multiplexed individual libraries (six per lane) and sequenced them as paired-end 50-bp reads on an Illumina HiSeq 2000 instrument at the Genome Sequencing Laboratory of the HudsonAlpha Institute. We used Cutadapt software (v. 1.8.1) to trim RNA-seq reads for residual adaptors and low quality sequences. Since a

551 good quality reference genome is not currently available for the striped mouse, we used a
552 dual exploratory strategy to assess differential expression between skin regions across
553 various developmental stages. First, we aligned the trimmed RNA-Seq reads against the
554 *Mus* reference genome version using genomic sequence and transcript annotations
555 obtained from Ensembl (release 80) and the STAR aligner software (v. 2.5.0b). In
556 parallel, we assembled trimmed RNA-Seq reads into a *de novo* transcriptome using the
557 Trinity suite of tools (v. 2.1.0). The resulting *de novo* transcriptome assembly was
558 subsequently annotated using an in-house annotation procedure supported by the human
559 reference exon sequences retrieved from the RefSeq sequence database (GRCh37/hg19,
560 release 55; http://www.ncbi.nlm.nih.gov/refseq/). Briefly, to associate a specific gene
561 entity to each *de novo* assembled transcriptomic contig, we mapped our *de novo* assembly
562 against a comprehensive database of reference human exon sequences using the Blast
563 software (v. 2.2.22). We retained only alignments with a significant Blast Expected Value
564 $< 10^{-4}$ for subsequent annotation purposes. Based on these alignments, we subsequently
565 computed an *ad-hoc* mapping score for each pair {assembly contig, gene entity} for
566 which at least one significant exon alignment was identified. The mapping score was
567 computed as the sum of the highest Blast alignment bit-scores at each position within a
568 particular contig, associated with at least one significant alignment against an exon of the
569 considered gene entity. Ultimately, the annotation procedure associated to each mapped
570 contig the gene entity with the highest *ad-hoc* mapping score. We then used the annotated
571 *de novo* transcriptome assembly as reference for aligning trimmed RNA-Seq reads using
572 the Bowtie2 software (v. 2.2.5).

573 We computed gene counts from read alignments, obtained using either the *Mus*
574 reference or the *de novo* transcriptome assembly, with three software tools included in the
575 Trinity suite: eXpress (v. 1.5.1), Kallisto (0.42.4) and RSEM (v. 1.2.23). We then used
576 the individual sets of gene counts computed for each transcriptome reference and each
577 abundance estimation tool to test for differential gene expression between samples from
578 various skin regions with the DESeq2 package (v. 1.10.1) from Bioconductor. The entire
579 dataset (3 individuals, 3 regions, 4 stages; n=36 libraries) was analyzed under the
580 DESeq2 negative binomial generalized linear model, which is a powerful and robust
581 approach for identifying genes that are differentially expressed, either between stages or
582 between regions. In all analyses, we used a false discovery ratio (FDR) <0.1 as a
583 statistical significance threshold. Results presented here depict region-specific two-way
584 comparisons across all four stages, dark vs. light (Fig. 2a), dark vs. flank (Extended Data
585 Fig. 4e), and flank vs. light (Extended Data Fig. 4f), in which genes assessed as
586 significant represent the intersection between the three abundance estimation approaches
587 implemented in the Trinity suite.
588 There are major changes in cell composition and skin development across the four
589 stages we examined by RNA-Seq (E19, E22, P0, P2), associated with large changes in
590 gene expression profiles. Therefore to examine the relationship between light or dark
591 stripes at different stages, we developed supervised learning models in which the gene
592 expression profile at one stage was tested as a predictor of stripe identity, light vs. dark,
593 and subsequent stages. After normalization and variance stabilization using R functions
594 implemented in DESeq2, we carried out a principal component analysis (PCA), using R
595 functions implemented in the FactoMineR (v.1.33) package, to identify variance

components associated with light vs. dark phenotype across all stages. The PCA results then were used to develop supervised learning models using the R functions implemented in the randomForest package (v.4.6-12), including optimization steps based on the top 5% of the most informative gene expression profiles associated with each stage. The results demonstrate the ability of learning models based on a specific region and stage to predict region identity, light vs. dark, or other stages, in which accuracy of the models is evaluated by averaging over 30 independent iterations (Extended Data Fig. 2f).

***In situ* hybridizations**

For *in situ* hybridization, we generated species-specific riboprobes by cloning a 545bp fragment of *Alx3* from *Mus* and the striped mouse. We carried out section *in situ* hybridizations following protocols described previously[39,40] and visualized samples using an A1 Imager (Zeiss). All pictures are representative of at least three individuals.

**Cell culture experiments**

We purchased B16-F1 melanoma cells from ATCC and maintained them in DMEM with 10% Fetal Bovine Serum (FBS, Sigma-Aldrich), 100 U/mL penicillin, and 100 μg/mL streptomycin in a 37$^{o}$C incubator with 5% $CO_2$ at physiological pH 7.4. We grew cells to 70-80% confluency and performed all experiments within 10 passages. B16-F1 cells tested negative for *Mycoplasma* contamination. We cultured mouse keratinocytes and maintained them in 0.05 mM $Ca^{2+}$ E-media with 10% FCS serum, following previously established protocols[18].

For gain-of-function experiments, we used the LV-*Alx3*:GFP and LV-GFP constructs described above. For loss-of-function experiments, we used five constructs (four specific to *Alx3* and one scrambled sequence) from existing RNAi lentiviral libraries[41] (details and clone numbers listed in Supplementary Table 3). For viral infections, we plated cells in 6-well dishes at 300,000 cells per well and incubated with lentivirus in the presence of polybrene (100μg/mL). All infections were carried in triplicate. After two days in culture, we selected infected cells using either puromycin (2μg/mL; shRNA constructs) or FACS (gain-of-function), and processed samples for mRNA analyses. For cell culture insert experiments, we plated wildtype cells on 0.4 um transwell inserts (Falcon, BD) at 200,000 cells/mL and incubated them in plates containing a bottom layer of transduced cells (keratinocytes or melanocytes) for three days (see Extended Data Fig. 7a, c for an illustration of the experimental design).

**Ultrasound-assisted *in utero* lentiviral microinjections**

For construction of LV-*Alx3*:GFP, we replaced the puromycin cassette of PLKO.1, a generic lentiviral vector containing the PKG promoter[41], with a fragment containing *Alx3* cDNA cloned from *Mus* (NM_007441.3), a P2A sequence, and a histone-fluorescent protein gene fusion (Hist2h2be-eGFP). LV-GFP, which contains only the sequence coding for Hist2h2be-eGFP, was originally designed from PLKO.1 using a similar strategy[18] (Addgene plasmid 25999). We carried out large-scale production of VSV-G pseudotyped lentivirus using calcium phosphate transfections of 293FT cells and helper plasmids, pMD2.G and psPAX2 (Addgene plasmids 12259 and 12260). Transfection conditions, subsequent viral concentration, and titering followed established

641 guidelines[18]. For injections, we anesthetized C57Bl6 females at E8.5 of gestation and
642 injected embryos with 1.5 µl of a constant viral titer. We collected transduced skin from
643 P4 pups, which we used for immunohistochemistry, following procedures outlined above,
644 and for isolation of virus infected melanocytes via FACS. For FACS, we used a KIT
645 antibody (ebioscience 14-1171-81; 1:1000), sorted $KIT^{+}GFP^{+}$ cells directly in Trizol LS
646 (Invitrogen), and extracted RNA following the protocol outlined in the TRIZOL LS
647 manual. We manually quantified the number of $MITF^{+}GFP^{+}$ and $SOX10^{+}GFP^{+}$ cells in
648 hair follicles, as detected with our antibodies. To determine the number of keratinocytes
649 per follicular area, we divided the number of $K14^{+}GFP^{+}$ cells in hair follicles by the hair
650 bulb area, as determined using ImageJ[35]. To determine hair follicle density, we obtained
651 pictures of skin sections stained with DAPI and counted the number of hair follicles per
652 tissue section area, using ImageJ[35]. Data were obtained from three individuals per
653 lentiviral construct injected. All counts were done blind. Statistical differences were
654 established using two-tailed *t* tests (sample sizes for each analysis are indicated in figure
655 legends).
656

657 **Comparative sequence analysis**

658 We obtained comparative sequence data from publicly available nucleotide
659 databases at NCBI (http://www.ncbi.nlm.nih.gov/BLAST/). Evolutionarily conserved
660 non-coding sequences were identified using the global sequence alignment tool
661 incorporated in the UCSC genome browser (http://genome.ucsc.edu)[42], PipMaker
662 (http://bio.cse.psu.edu/pipmaker)[43], and LAGAN
663 (http://lagan.stanford.edu/lagan_web/index.shtml)[44].

664

665 **Electrophoretic mobility shift assays (EMSA)**

666 We conducted EMSA using nuclear extracts[45] of melanoma B16-F1 cells
667 prepared in the presence of protease inhibitors (Complete protease inhibitor cocktail;
668 Roche) and determined protein concentrations using the Bio-Rad protein assay. Synthetic
669 complementary oligonucleotides were annealed and labeled using [$\gamma$-$^{32}$P] ATP and T4
670 kinase. We performed binding reactions at room temperature in the presence of 20,000
671 cpm of radiolabeled probe (approximately 6–10 fmol) in a volume of 20 µl containing 2
672 µg poly (dI-dC), 20 mM HEPES (pH 7.9), 70 mM KCl, 2.5 mM MgCl2, 1 mM
673 dithiothreitol, 0.3 mM EDTA, and 10% glycerol. We then added competitor
674 oligonucleotides of identical (specific) or unrelated (non-specific) sequence to the probe
675 at the indicated fold molar excess. The sequences of the oligonucleotides used are listed
676 in Supplementary Table 2. When indicated, we added specific antiserum[24] or control non-
677 immune rabbit serum to the binding reaction. The reaction mixtures were resolved using
678 5% nondenaturing polyacrylamide gels, which were subsequently dried and
679 autoradiographed.

680

681 **Western Blots**

682 We prepared cell lysates from B16-F1 cells, resolved by SDS/PAGE, and blotted
683 them onto a BioTrace PVDF membrane (Pall Corporation). We detected ALX3
684 immunoreactivity with a rabbit polyclonal primary antiserum (1:4000 dilution)[24] and a
685 horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (1:10000 dilution;
686 Bio-Rad Laboratories). To detect ACTIN we used a mouse monoclonal antibody

687 (1:10000 dilution, clone AC-15; Sigma) and a horseradish peroxidase-conjugated goat
688 anti-mouse antibody (1:5000 dilution; Bio-Rad Laboratories). We visualized
689 immunoreactive bands using an ECL detection system (GE Healthcare).
690
691 **Chromatin immunoprecipitation (ChIP) – qPCR assays.**
692 We performed ChIP assays as previously described[46] using B16-F1 cells treated
693 with 1% formaldehyde. We isolated the cross-linked protein-DNA complexes and, after
694 sonication, we incubated chromatin with an ALX3 antiserum[24] or with control non-
695 immune rabbit serum. Next, we isolated antibody-protein-DNA complexes by incubation
696 with protein A-Sepharose. To detect bound DNA, we carried out qPCR out on triplicate
697 samples using oligonucleotide primers that amplify fragments of the *Mitf* gene
698 corresponding to the regions containing either Site 3, 5, and 10. As a control, we used
699 promoter sequences from the *Tyr* gene as described[47]. Oligonucleotides used in ChIP
700 assays are listed in Supplementary Table 2.
701
702 **Luciferase assays**
703 We amplified a 1.5kb region of the *Mitf* M promoter from *Mus* and subsequently
704 cloned it into the SacI-HindIII sites of the pLightSwitch_Prom luciferase reporter vector
705 (Switchgear Genomics, Active Motif). We then generated additional luciferase constructs
706 from the wild-type construct by mutating the different *Alx3* binding motifs (TAAT for
707 GCCG) using the Q5 Site-Directed Mutagenesis Kit (New England Biolabs). We verified
708 all constructs by sequencing. We next transfected B16-F1 melanocytes with LV-
709 *Alx3*:GFP and LV-GFP by using FuGENE HD (Active Motif). Using FACS, we selected

710 the stable transfectant clones and confirmed overexpression of *Alx3* by qPCR. The day
711 prior to the transfection, we seeded cells at a density of $1x10^4$ cells per well and 16 hours
712 later transfected them with the different *Mitf* constructs using a FuGENE HD to plasmid
713 DNA ratio of 3:1 (300nL FuGENE HD to 100ng plasmid DNA per well). We then
714 harvested cells and processed them using the LightSwitch Luciferase Assay Kit
715 (Switchgear Genomics) following the protocol guidelines and measured luciferase using
716 a SpectraMax L luminometer (Molecular Devices). We normalized luciferase activity
717 relative to luminescence from cells transfected with the pLightSwitch_Prom luciferase
718 reporter vector (empty vector). We did not observe a difference in luciferase activity
719 when we transfected our two stable cell lines (LV-*Alx3*:GFP and LV-GFP) with an empty
720 vector (pLightSwitch_Prom) or a vector containing the promoter for a housekeeping gene
721 (ACTB_PROM). We performed all luciferase experiments using five replicates per
722 construct and established statistical significance of luminescence differences using two-
723 tailed *t* tests (sample sizes for each experiment are indicated in figure legends).
724
725 **Chipmunk (*Tamias striatus*) samples**
726 We collected *Tamias striatus* at Harvard University's Concord Field Station
727 (Concord, MA) using Sherman live traps (Massachusetts state permit: 027.14SCM).
728 Chipmunks were euthanized, skin punches were taken from the different body regions,
729 and samples were processed for qPCR as indicated above.
730
731 **Extended Data Figure legends**

**Extended Data Figure 1. Hair characterization in adult striped mice. a,** Striped mice have three different phenotypic categories of hair (*light, black, and banded*) based on individual pigment pattern. All hair types have a black tip, which corresponds to structural hair features (not pigment). **b,** Relative proportion of *light*, *black*, and *banded* guard and awl hair along the striped mouse dorsoventral axis ($n$ = 5; error bars represent SEM). Scale bar in (**a**) 100 μm.

**Extended Data Figure 2. Stripe-like differences in hair length along the dorsum in striped mice embryos and pups. a-c,** Flat-mount skin preparations ('dermis up') of embryos at E16 (**a**), E19 (**b**), and pups from P2 (**c**). Middle axis is indicated in all cases (midline). White dashed lines demarcate regions differing in hair length at E19 (**b**) and regions differing in pigmentation at P2 (**c**). Incipient pigmentation stripes are shown in (**b**). **d,** Skin punches (1mm) and length measurements show differences between hair in the dark and light stripe of P2 individuals. Hair length differences in (**b**) (incipient stripes) correlate with those seen when pigment differences arise (**c, d**). Differences among dorsal regions were evaluated by ANOVA followed by a Tukey-Kramer test; $n$ = 15 per region; statistically significant differences ($P < 0.05$) are indicated by different letters. Red bars indicate the mean. **e,** Hair length measurements taken from guard, awl, and zigzag hair found along the dorsum of adults. Differences among dorsal regions were evaluated by ANOVA followed by a Tukey-Kramer test; $n$ = 24 (Guard), 12 (Awl), and 12 (Zigzag) per region; $P$ = 0.1736 (guard hair), $P$ = 0.8006 (awl hair), $P$ = 0.1038 (zigzag hair). **f,** Predicted probabilities of the observed stripe-like phenotypes, as inferred by supervised learning models built and trained to recognize time-point-specific gene

755 expression signatures of the stripes. Bars reflect average probabilities (+ SEM) computed
756 from 30 consecutive iterations of the predictive model in each analysis. Labels indicate,
757 as a ratio, the time point at which the randomForests model was applied to predict the
758 stripe-like phenotype (the left term of the ratio), and the time point at which the model
759 was built and trained (the right term of the ratio). Dotted line indicates the prior
760 probability of either stripe phenotype (i.e., 50% in case a case with only two distinct
761 phenotypes). Please see Methods for details.
762
763 **Extended Data Figure 3. Cell proliferation and hair follicle density in postnatal day**
764 **2 (P2) striped mice. a,** Counts of proliferating cells, as determined by EdU labeling, in
765 the epidermis and inside hair follicles (epidermal cells: dark stripe 1 vs. light stripe, two-
766 tailed *t* test; $n = 15$ images per region, $P = 0.5417$; cells counted: 402 [dark stripe 1] and
767 444 [light stripe]; intrafollicular cells: dark stripe 1 vs. light stripe, two-tailed *t* test; $n =$
768 12 images per region, $P = 0.7537$; cells counted: 724 [dark stripe 1] and 680 [light
769 stripe]). **b,** Number of hair follicles per surface area along the dorsoventral axis
770 (differences among dorsal regions were evaluated by ANOVA; $n = 10$ images per region,
771 $P = 0.4391$; number of hair follicles counted: 139 [light stripe], 141 [dark stripe 1], 132
772 [dark stripe 2], and 128 [flank]). Bright field images in (**a**) depict pigment. Red bars
773 indicate the mean. Scale bars in (**a**) 100 μm, (**b**) 200 μm.
774
775 **Extended Data Figure 4**. **RNA-Seq analysis. a-d,** Venn diagrams showing numbers of
776 differentially expressed genes identified using either the *Mus* reference or the striped
777 mouse *de novo* transcriptome assembly in light vs. dark stripes (**a**), light vs. flank (**b**), and

778 flank vs. dark (**c**), or differentially expressed genes in light or dark stripes vs. the other
779 skin region (light or dark stripes and the flank) (**d**). Genes that are specifically
780 upregulated only in the dark or light stripes are highlighted in red. **e, f,** RNA-Seq
781 transcript levels (normalized gene counts) plotted as a function of differential expression
782 ($\log_2$ fold-change). The 1148 genes demonstrating significant (FDR < 0.1) differential
783 expression in the flank versus the dark stripe are shown in yellow (higher expression in
784 the flank) or blue (higher expression in the dark stripe) (**e**). Eleven differentially
785 expressed pigmentation-related genes are highlighted (dark yellow or dark blue), while 6
786 additional pigmentation-related genes that are not differentially expressed are shown in
787 pink (**e;** Supplementary Table 1c). The 1777 genes demonstrating significant (FDR < 0.1)
788 differential expression in the light stripe versus the flank are shown in yellow (higher
789 expression in the light stripe) or blue (higher expression in the flank) (**f**). Four
790 differentially expressed pigmentation-related genes are highlighted (dark yellow or dark
791 blue), while 11 additional pigmentation-related genes that are not differentially expressed
792 are shown in pink (**f**; Supplementary Table 1b).
793
794 **Extended Data Figure 5. Stage-specific gene expression. a-c,** Quantitative PCR shows
795 the relative mRNA levels of pigment-type switching genes *Asip* (**a**), *Edn3* (**b**), and
796 melanin synthesis genes *Tyr* and *Tyrp1* (**c**) in different regions of the striped mouse skin
797 and at different time points. Differences among stripes in within each time point was
798 evaluated by ANOVA followed by a Tukey-Kramer test; $n = 4$ per time point;
799 statistically significant differences ($P < 0.05$) are indicated by different letters. Red bars
800 indicate the mean.

**Extended Data Figure 6. Gain- and loss-of function experiments in cultured cells. a,** Lentiviral constructs were modified from pLKO.1, a generic vector for expressing human RNU6-1 promoter-driven short hairpin RNAs (red loop). LTR, long terminal repeat; ψ, retroviral packaging element, RRE, Rev response element; cPPT, central polypurine tract; PGK, phosphoglycerate kinase promoter; H2B-GFP, Hist2h2be fused to GFP cDNA; P2A, 2A peptide. **b,** Western blot shows expression of ALX3 in nuclear extracts of B16-F1 cells. Positive controls were extracts from mouse embryonic mesenchyme (MEM) or COS cells transfected with a pcDNA-ALX3 expression vector. COS cells transfected with empty pcDNA served as negative controls. ACTIN immunoreactivity is shown below for the same extracts as a control. For gel source data, see Supplementary Figure 1. **c, d,** Quantitative PCR shows *Alx3* (white), *Mitf* (black), and *Silver* (grey) mRNA levels in cells transduced with (**c**) LV-*Alx3*:GFP, relative to cells transduced with the LV-GFP control (LV-*Alx3*:GFP vs. LV-GFP, two-tailed *t* test; $n = 3$) or (**d**) shRNA lentiviral constructs, relative to cells transduced with a scrambled control (shRNA1, 2, 3, or 4 vs. shRNA scrambled, two-tailed *t* test; $n = 3$). Statistically significant differences [$P < 0.05$] are indicated by different letters. Red bars indicate the mean.

**Extended Data Figure 7. Co-culture experiments. a-d,** Wildtype B16 melanocytes (B16 WT) were exposed to keratinocytes (**a**) or melanocytes (**c**) stably transduced with either LV-*Alx3*:GFP (LV-*Alx3* in graphs) or LV-GFP. **b, d,** Quantitative PCR shows levels of *Alx3* mRNA in cells carrying the lentiviral constructs (gray panel) and of *Mitf*, *Tyr*, and *Silver* mRNA in B16 WT melanocytes exposed to keratinocytes (**c**) or

melanocytes (**d**) transduced with LV-*Alx3*:GFP (red panels) or LV-GFP (blue panels) (LV-*Alx3*:GFP vs. LV-GFP, two-tailed *t* test; $n = 3$, $^{***}P < 0.0001$) Red bars in (**b**) and (**d**) indicate the mean.

**Extended Data Figure 8. Ultrasound-guided *in utero* lentiviral injections. a-f**, Hair follicles from embryos injected with lentiviruses stained for SOX10 (**a, d**), virus transduced cells (**b, e**) and merged images with arrowheads showing $SOX^+GFP^+$ cells (**c, f**). **g**, Number of detectable $SOX10^+$ cells (LV-*Alx3*:GFP vs. LV-GFP, two-tailed *t* test; $n = 60$, $P = 0.1173$; cells counted: 426 cells [LV-*Alx3*:GFP] and 398 cells [LV-GFP]). **h-o**, Effect of *Alx3* on skin. Hair follicles from samples injected with LV-GFP control and LV-*Alx3*:GFP depicting immunohistochemistry for K14 (**h, k**), virus transduced cells (**i, l**), and merged images showing $K14^+GFP^+$ cells (**j, m**). **n,** Number of detectable $K14^+GFP^+$ cells per follicular area (LV-*Alx3*:GFP vs. LV-GFP, two-tailed *t* test; $n = 40$, $P = 0.275$; Average: 52.809 cells/hair follicle area [LV-*Alx3*:GFP] and 55.123 cells/hair follicle area [LV-GFP]). **o,** Hair follicle density in samples injected with viruses (LV-*Alx3*:GFP vs. LV-GFP, two-tailed *t* test; $n = 30$, $P = 0.103$; Average: 0.794 hair follicles/surface area [LV-*Alx3*:GFP] and 0.84 hair follicles/surface area [LV-GFP]). Scale bars in (**a-f** and **h-m**) 50 μM.

**Extended Data Figure 9. Alignment of a ~1.5kb region of the *Mitf* M promoter in *Mus* and striped mouse.** Black boxes represent conserved sequences. Mapped onto the sequences are evolutionary conserved regions of the mammalian *Mitf* M promoter identified *in silico* (http://genome.ucsc.edu) (yellow), regions from which the EMSA

probes were designed (red), and the transcription start site (green). The ten TAAT binding sites, conserved between *Mus* and striped mice (blue), that were tested are labeled sequentially.

**Extended Data Figure 10**. **EMSA and luciferase assays. a,** EMSAs show the binding of nuclear proteins from B16-F1 cells to candidate-binding sites 3, 5, and 10. The absence (-) or presence of non-specific (NSC; 500-fold molar excess) or specific (SC; indicated fold molar excess) competing oligonucleotides, or the addition of ALX3 antibodies or control (non-immune rabbit serum [NRS] or IgG) is indicated. Arrows indicate complexes containing ALX3, arrowhead shows supershift for site 3, and asterisk highlights an artifact in the gel. **b,** EMSAs showing the binding of recombinant *Alx3* synthesized using a rabbit reticulocyte lysate system to the indicated sites (sites 4-9). The absence (-) or presence of non-specific (NSC) or specific (SC) competing oligonucleotides (500-fold molar excess) is indicated. Note that site 5 is the only one showing sequence-specific binding. For gel source data, see Supplementary Figure 1. **c,** Relative levels of luciferase activity in B16-F1 cells stably expressing GFP (dark circles) or *Alx3* (light circles) for indicated binding sites. Luciferase activity was normalized relative to cells transfected with the empty reporter vector and values are given as a fraction of luminescence for GFP transfected cells. Differences between cells transfected with LV-*Alx3*:GFP and LV-GFP for each plasmid was evaluated using two-tailed t tests; $n = 5$; $*P < 0.05$. Red bars in indicate the mean. Labels of mutated binding sites correspond to those described in Fig. 4a.

## Supplementary Information

Supplementary material includes Supplementary Tables 1-3 and Supplementary Figure 1.

## Competing interests

The authors have no competing interests

## Data deposition:

Striped mouse *de novo* transcriptome assembly has been deposited in: https://datadryad.org/resource/doi:10.5061/dryad.7v222


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## Author contributions

RM, MM[5], and HEH conceived the project. RM, GSB, and HEH designed experiments. RM performed all experiments with the exception of: RM and MM[5] collected samples and performed phenotypic characterization; RM and SB performed *in*

*vivo* lentiviral injections; $MM^4$ and MV performed protein-DNA binding assays. CH carried out the large-scale RNA experiments, including construction and annotation of the *de novo* transcriptome, and design and analysis of the RNA-seq work. CS provided the first embryos for pilot studies and founding members for the striped mice lab colony. RM, GSB, and HEH wrote the paper with input from all authors.